

## University of Dundee

### **A spontaneous increase in intracellular Ca<sup>2+</sup> in metaphase II human oocytes in vitro can be prevented by drugs targeting ATP-sensitive K<sup>+</sup> channels**

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# A spontaneous increase in intracellular $\text{Ca}^{2+}$ in metaphase II human oocytes *in vitro* can be prevented by drugs targeting ATP-sensitive $\text{K}^+$ channels

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**STUDY QUESTION:** Could drugs targeting ATP-sensitive  $\text{K}^+$  ( $\text{K}_{\text{ATP}}$ ) channels prevent any spontaneous increase in intracellular  $\text{Ca}^{2+}$  that may occur in human metaphase II (MII) oocytes under *in vitro* conditions?

**SUMMARY ANSWER:** Pinacidil, a  $\text{K}_{\text{ATP}}$  channel opener, and glibenclamide, a  $\text{K}_{\text{ATP}}$  channel blocker, prevent a spontaneous increase in intracellular  $\text{Ca}^{2+}$  in human MII oocytes.

**WHAT IS KNOWN ALREADY:** The quality of the oocyte and maintenance of this quality during *in vitro* processing in the assisted reproductive technology (ART) laboratory is of critical importance to successful embryo development and a healthy live birth. Maintenance of  $\text{Ca}^{2+}$  homeostasis is crucial for cell wellbeing and increased intracellular  $\text{Ca}^{2+}$  levels is a well-established indicator of cell stress.

**STUDY DESIGN, SIZE, DURATION:** Supernumerary human oocytes ( $n = 102$ ) collected during IVF/ICSI treatment that failed to fertilize were used from October 2013 to July 2015. All experiments were performed on mature (MII) oocytes. Dynamics of intracellular  $\text{Ca}^{2+}$  levels were monitored in oocytes in the following experimental groups: (i) Control, (ii) Dimethyl sulfoxide (DMSO; used to dissolve pinacidil, glibenclamide and 2,4-Dinitrophenol (DNP)), (iii) Pinacidil, (iv) Glibenclamide, (v) DNP: an inhibitor of oxidative phosphorylation, (vi) Pinacidil and DNP and (vii) Glibenclamide and DNP.

**PARTICIPANTS/MATERIALS/SETTINGS/METHODS:** Oocytes were collected under sedation as part of routine treatment at an assisted conception unit from healthy women (mean  $\pm$  SD) age  $34.1 \pm 0.6$  years,  $n = 41$ . Those surplus to clinical use were donated for research. Oocytes were loaded with Fluo-3  $\text{Ca}^{2+}$ -sensitive dye, and monitored by laser confocal microscopy for 2 h at 10 min intervals. Time between oocyte collection and start of  $\text{Ca}^{2+}$  monitoring was  $80.4 \pm 2.1$  h.

**MAIN RESULTS AND THE ROLE OF CHANCE:** Intracellular levels of  $\text{Ca}^{2+}$  increased under *in vitro* conditions with no deliberate challenge, as shown by Fluo-3 fluorescence increasing from  $61.0 \pm 11.8$  AU (AU = arbitrary units;  $n = 23$ ) to  $91.8 \pm 14.0$  AU ( $n = 19$ ;  $P < 0.001$ ) after 2 h of monitoring. Pinacidil (100  $\mu\text{M}$ ) inhibited this increase in  $\text{Ca}^{2+}$  ( $85.3 \pm 12.3$  AU at the beginning of the experiment,  $81.7 \pm 11.0$  AU at the end of the experiment;  $n = 13$ ;  $P = 0.616$ ). Glibenclamide (100  $\mu\text{M}$ ) also inhibited the increase in  $\text{Ca}^{2+}$  ( $74.7 \pm 10.6$  AU at the beginning and  $71.8 \pm 10.9$  AU at the end of the experiment;  $n = 13$ ;  $P = 0.851$ ). DNP (100 mM) induced an increase in intracellular  $\text{Ca}^{2+}$  that was inhibited by glibenclamide (100  $\mu\text{M}$ ;  $n = 9$ ) but not by pinacidil (100  $\mu\text{M}$ ;  $n = 5$ ).

**LIMITATIONS, REASONS FOR CAUTION:** Owing to clinical and ethical considerations, it was not possible to monitor  $\text{Ca}^{2+}$  in MII oocytes immediately after retrieval. MII oocytes were available for our experimentation only after unsuccessful IVF or ICSI, which was, on average,  $80.4 \pm 2.1$  h ( $n = 102$  oocytes) after the moment of retrieval. As the MII oocytes used here were those that were not successfully fertilized, it is possible that they may have been abnormal with impaired  $\text{Ca}^{2+}$  homeostasis and, furthermore, the altered  $\text{Ca}^{2+}$  homeostasis might have been associated solely with the protracted incubation.

**WIDER IMPLICATIONS OF THE FINDINGS:** These results show that maintenance of oocytes under *in vitro* conditions is associated with intracellular increase in  $\text{Ca}^{2+}$ , which can be counteracted by drugs targeting  $\text{K}_{\text{ATP}}$  channels. As  $\text{Ca}^{2+}$  homeostasis is crucial for contributing to a successful outcome of ART, these results suggest that  $\text{K}_{\text{ATP}}$  channel openers and blockers should be tested as drugs for improving success rates of ART.

**STUDY FUNDING/COMPETING INTEREST(S):** University of Dundee, MRC (MR/K013343/1, MR/012492/1), NHS Tayside. Funding NHS fellowship (Dr Sarah Martins da Silva), NHS Scotland. The authors declare no conflicts of interest.

**Key words:** human oocytes /  $\text{Ca}^{2+}$  / ATP-sensitive  $\text{K}^{+}$  channels / pinacidil / glibenclamide

## Introduction

Approximately 1 in 7 couples of reproductive age are classed as infertile, equating to ~72 million people globally. The primary treatment is assisted reproductive technology (ART) consisting of IVF and ICSI. However, despite the rapid developments in ART over the last decade, current success rates remain both sub-optimal and variable. For example, in the European Union the average IVF pregnancy rate is 29.2% per aspiration—ranging from 21.5 to 48.1% (Kupka et al., 2014). Even in oocyte donors, it is calculated that less than 7% of the oocytes produce a live birth—a figure that has shown little improvement in the last 12 years (Patrizio and Sakkas, 2009).

It is well established that the quality of the egg and maintenance of this quality during *in vitro* processing in the ART laboratory is of critical importance to successful embryo development and a healthy live birth (reviewed by Marteil et al., 2009; Lord and Aitken, 2013). Additionally, there are recent reports suggesting that animals generated by IVF exhibit vascular dysfunction later in life and shortened lifespan in general. It has been proposed that stress associated with ART *in vitro* procedures is most likely responsible for this phenomenon (Rexhaj et al., 2013). Removal of an egg from its natural environment (the follicle) and exposure to *in vitro* conditions is a considerable stress (Khosla et al., 2001). Although there is wide variation in ART laboratory practice, following follicle aspiration eggs can be left for several (~2–6) hours and, in the case of ICSI, stripped of their cumulus before injection/addition of sperm. Although handling and manipulation of the eggs in the laboratory aims to minimize external stresses, data from work on oocytes of experimental animals and many other cell types indicate that cellular stress might occur (Takahashi et al., 2009). An important effect of *in vitro* stress is likely to be disturbance of ionic regulation of the cell. For instance, oxidative stress ( $\text{H}_2\text{O}_2$ ) and *in vitro* ageing of metaphase II (MII) eggs in mice induces impairment of calcium homeostasis and poor subsequent embryo development (Takahashi et al., 2009). Higher intracellular  $\text{Ca}^{2+}$  is observed with oxidative stress, and evidence of impaired store-operated calcium entry is found in *in vitro* aging (24 h) (Martín-Romero et al., 2008). An increase in intracellular  $\text{Ca}^{2+}$  has been recognized as a trigger of events that ultimately lead to cell death. However, in all studies addressing  $\text{Ca}^{2+}$  homeostasis in oocytes *in vitro*, stress was induced by different means. Whether  $\text{Ca}^{2+}$  levels in oocytes remain steady under routine *in vitro* conditions when there is no deliberate induction of stress is yet unknown. Any change in  $\text{Ca}^{2+}$  dynamics has

the potential to affect intracellular signalling and could be associated with unsuccessful fertilization and/or health issues later in life.

The regulation of  $\text{Ca}^{2+}$  homeostasis is crucial for cellular protection during stress (Clapham, 2007), and impaired  $\text{Ca}^{2+}$  homeostasis in oocytes negatively impacts fertilization and development (Miao et al., 2009). ATP-sensitive  $\text{K}^{+}$  ( $\text{K}_{\text{ATP}}$ ) channels are  $\text{K}^{+}$ -selective channels gated by intracellular ATP. As such, they are suggested to be a link between intracellular metabolic conditions and cellular membrane excitability. In tissues where they are expressed,  $\text{K}_{\text{ATP}}$  channels play a crucial physiological role, including a role in insulin secretion, appetite control, smooth muscle tone and others (reviewed by Nichols, 2006). Additionally, regulation of channel activity and numbers has been shown to regulate cellular resistance to different types of stresses in different types of cells (Crawford et al., 2002; Mohammed Abdul et al., 2014, 2015a,b). In oocytes,  $\text{K}_{\text{ATP}}$  channels have been recently identified (Du et al., 2010), but their physiological role is unknown. It is also unknown whether drugs targeting  $\text{K}_{\text{ATP}}$  channels would have any effect on oocyte function. If  $\text{Ca}^{2+}$  homeostasis is challenged under routine ART laboratory conditions, oocyte intracellular signalling pathways may be sufficiently compromised so as to reduce the chances of successful conception without any external signs to indicate this. Therefore, the aim of the present study was to establish whether intracellular  $\text{Ca}^{2+}$  levels change in human oocytes during incubation *in vitro* and, if it does, would drugs targeting  $\text{K}_{\text{ATP}}$  channels counteract such changes.

## Materials and Methods

### Human oocytes

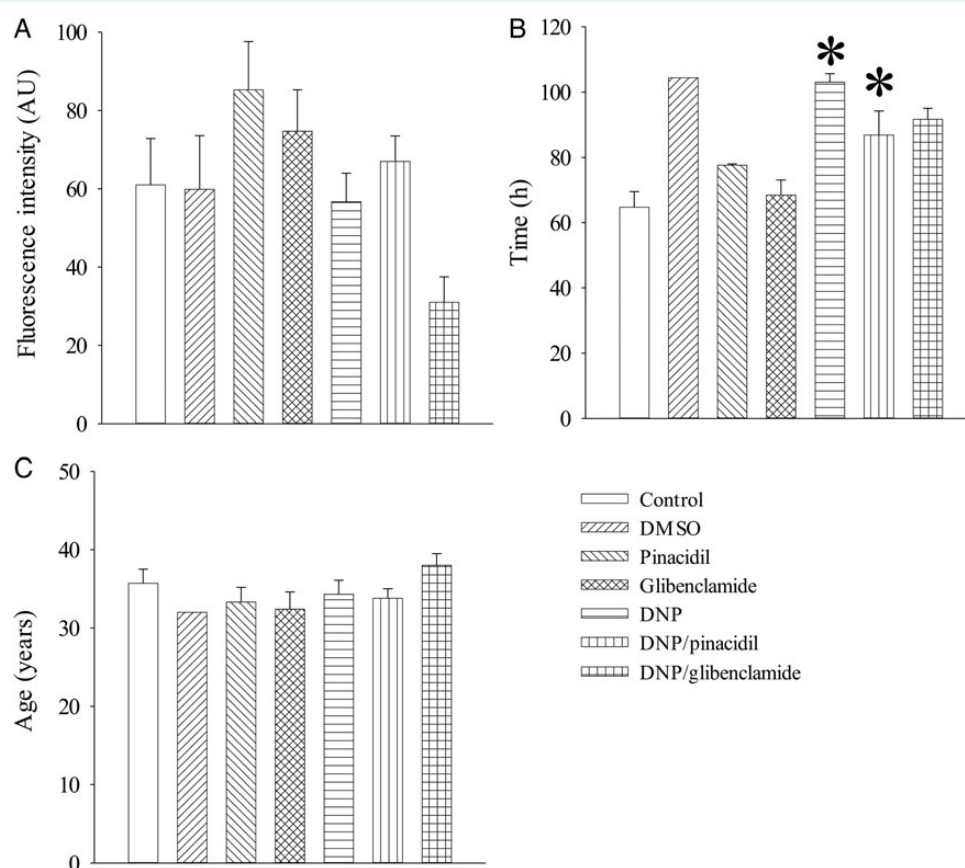
All experiments were performed on supernumerary human mature (MII) oocytes. Oocytes were collected from 41 healthy women (average age  $34.1 \pm 0.6$  years) undergoing assisted reproduction treatment at Ninewells Assisted Conception Unit, Dundee, UK [HFEA centre # 0004]. The main reasons for infertility in donors were unexplained (38%) and male factor (29%), while only 3% had endometriosis, a condition that could potentially affect  $\text{Ca}^{2+}$  homeostasis in oocytes (Andrade et al., 2010; Carvalho et al., 2012; Singh et al., 2013). Ethical approval was provided by East of Scotland Research Ethics Service [number 08/S1402/23] and written informed consent was obtained from all women. MII oocytes used for this research had been inseminated (IVF) or injected (ICSI) for clinical treatment, but had failed to fertilize (no pronuclei and no cleavage). Oocytes were stripped for ICSI using ICSI Cumulase (Origio, Malov, Denmark), while oocytes for IVF

were not stripped but at fertilization check remaining cells were mechanically removed. From the point of assessment of fertilization to being donated for research, all oocytes were maintained in 20  $\mu\text{l}$  drops of cleavage medium under oil (SAGE; Cooper Surgical, Trimbull, CT, USA) at  $37^\circ\text{C}$  in 5%  $\text{O}_2$ , 6%  $\text{CO}_2$  balanced with nitrogen. Oocytes were arbitrarily divided between experimental groups that were studied independently from each other in order to assess  $\text{Ca}^{2+}$  levels: (i) Control (in the absence of any compound expected to influence intracellular  $\text{Ca}^{2+}$ ), (ii) Dimethyl sulfoxide (DMSO; at 0.1% DMSO, as used to dissolve pinacidil, glibenclamide and 2,4-dinitrophenol (DNP)), (iii) Pinacidil (100  $\mu\text{M}$  pinacidil, a  $\text{K}_{\text{ATP}}$  channel opener), (iv) Glibenclamide (100  $\mu\text{M}$  glibenclamide, a  $\text{K}_{\text{ATP}}$  channel blocker), (v) DNP (100  $\mu\text{M}$  DNP, an inhibitor of oxidative phosphorylation and inducer of chemical hypoxia), (vi) Pinacidil and DNP (both at 100  $\mu\text{M}$ ) and (vii) Glibenclamide and DNP (both at 100  $\mu\text{M}$ ). Unless indicated differently, cells in each experimental group were monitored continuously for 2 h. In a separate series of experiments, oocytes were loaded with Fluo-3 at  $37^\circ\text{C}$ . These oocytes were also used to make comparison between those exposed to IVF or ICSI. Pinacidil, glibenclamide, DNP and DMSO were purchased from Sigma-Aldrich, Gillingham, UK. There was no statistically significant difference in basal levels of intracellular  $\text{Ca}^{2+}$  between experimental groups (Fig. 1). Time from oocyte retrieval to start of  $\text{Ca}^{2+}$  monitoring was not significantly different for the control group compared with pinacidil and glibenclamide treatment

groups (Fig. 1). Female age of oocyte donors was also not significantly different between experimental groups (Fig. 1).

## Laser confocal microscopy and experimental protocol

MII oocytes for all experimental groups, apart from controls loaded at  $37^\circ\text{C}$ , were loaded (for 60 min) at room temperature with the esterified form of the  $\text{Ca}^{2+}$ -sensitive fluorescent probe, Fluo-3AM (5  $\mu\text{M}$  dissolved in 0.1% DMSO plus pluronic acid; Molecular Probes, Eugene, OR, USA). Afterwards, cells were superfused with collection solution (Sigma-Aldrich, M0393) with and without compounds targeting  $\text{K}_{\text{ATP}}$  channels (described in previous section) and imaged using laser confocal microscopy coupled to an inverted microscope (Leica TCS SP5 II, Milton Keynes, UK) with a  $\times 10$  (numerical aperture 1.3) oil-immersion objective lens at  $37^\circ\text{C}$ . The intensity of fluorescence of whole oocytes on the equatorial plane was measured. Microscope was calibrated by green calibration slide before each experiment. Intensity of fluorescence was described in arbitrary units (AU) covering a range from 0 to 60 000 AU.  $\text{Ca}^{2+}$  levels and cell morphology were imaged every 10 min for 2 h using an Argon/UV laser (excitation 488 nm/emission 520 nm). Images were analysed using Leica Application Suite AF Lite software (Leica). The parameters of image acquisition were similar for all examined cells. All compounds were purchased from Sigma-Aldrich. All obtained results were



**Figure 1** Age of donors, time between oocyte collection and experiments and basal level of  $\text{Ca}^{2+}$ . Basal levels of intracellular  $\text{Ca}^{2+}$  in human oocytes and age of donors of oocytes was not different between control and experimental groups while the time between oocyte collection and experiments was longer only in the DNP and DNP/pinacidil experimental groups when compared with the control. Average fluorescence at time point 0 (A), time passed between oocytes collection and experiment (B) and age of donors of oocytes (C) for control and experimental groups. Bars show the mean  $\pm$  SEM ( $n = 1-7$  for age and 5-23 for basal fluorescence and time passed between oocytes collection and experiment). \* $P < 0.05$ , ANOVA. AU, arbitrary units.

normalized in respect to the intensity of fluorescence at time point 0, that was considered to be 100%.

## Statistical analysis

Data are presented as mean  $\pm$  SEM. Mean values were compared using analysis of variance (ANOVA), ANOVA on ranks and the paired or unpaired t-test, where appropriate.  $P < 0.05$  was considered statistically significant. Statistical analysis was performed using SigmaPlot 12 (Systat Software, Hounslow, UK).

## Results

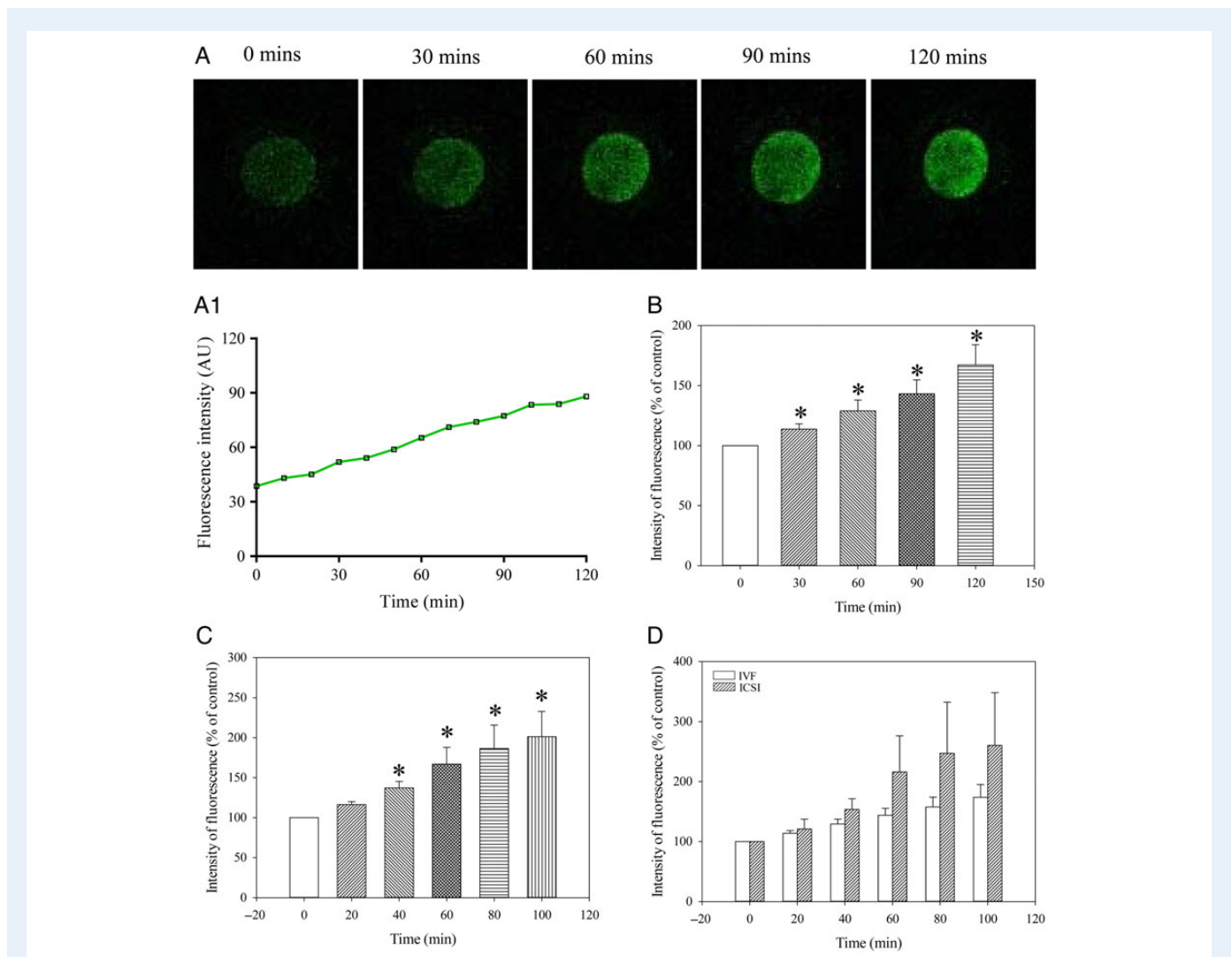
### Increase of $\text{Ca}^{2+}$ *in vitro* over time in human oocytes

$\text{Ca}^{2+}$  levels were captured every 10 min for 2 h. The increase in intensity of fluorescence reached statistical significance after 30 min (it increased

by  $13.7 \pm 4.5\%$  from the basal level),  $n = 19$ ,  $P = 0.007$ ; Fig. 2. After 120 min, intensity of fluorescence was increased by  $67.2 \pm 16.9\%$  from the basal level ( $n = 19$ ;  $P < 0.001$  compared with time 0; Fig. 2). Similar results were obtained when cells were loaded at  $37^\circ\text{C}$  (Fig. 2C). We have also examined whether there was a difference in fluorescence intensity among oocytes obtained from IVF versus ICSI patients. The mean change in fluorescence observed between the two sets of data was not statistically significant (Fig. 2D). DMSO (0.1%) did not have any effect on dynamics of intracellular  $\text{Ca}^{2+}$  under *in vitro* conditions (Fig. 3).

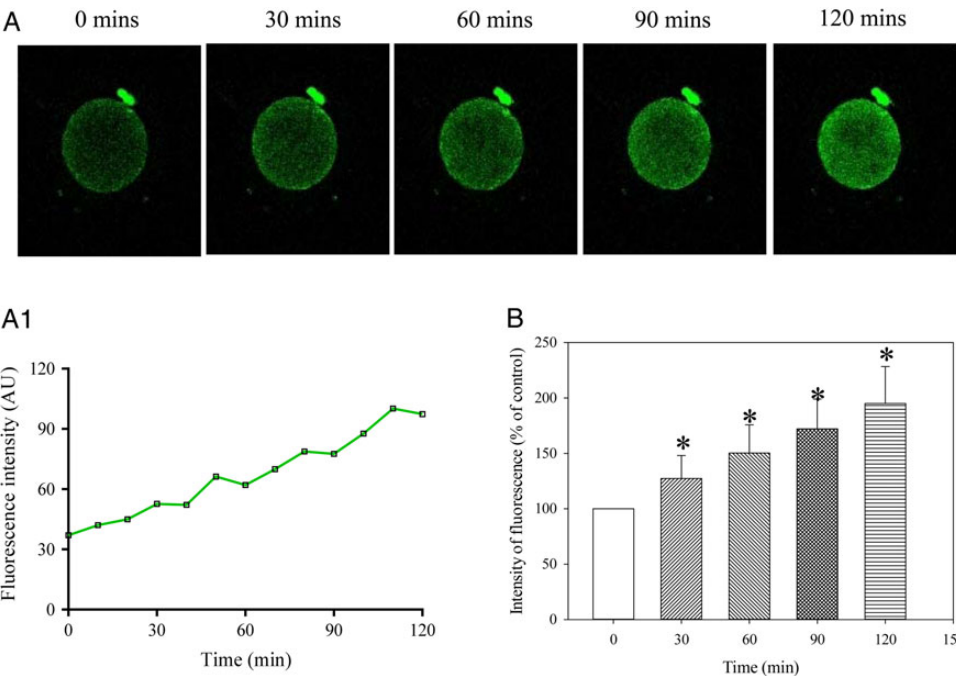
### Pinacidil inhibits the increase in $\text{Ca}^{2+}$ over time *in vitro* in human oocytes

In the presence of  $100 \mu\text{M}$  pinacidil, intracellular  $\text{Ca}^{2+}$  levels remain unchanged over time ( $P = 0.536$ ;  $n = 12-19$ ; Fig. 4). After 120 min, the intensity of fluorescence was  $99.9 \pm 8.9\%$  of that at time point 0 ( $P = 0.993$ ;  $n = 12-19$ ; Fig. 4).

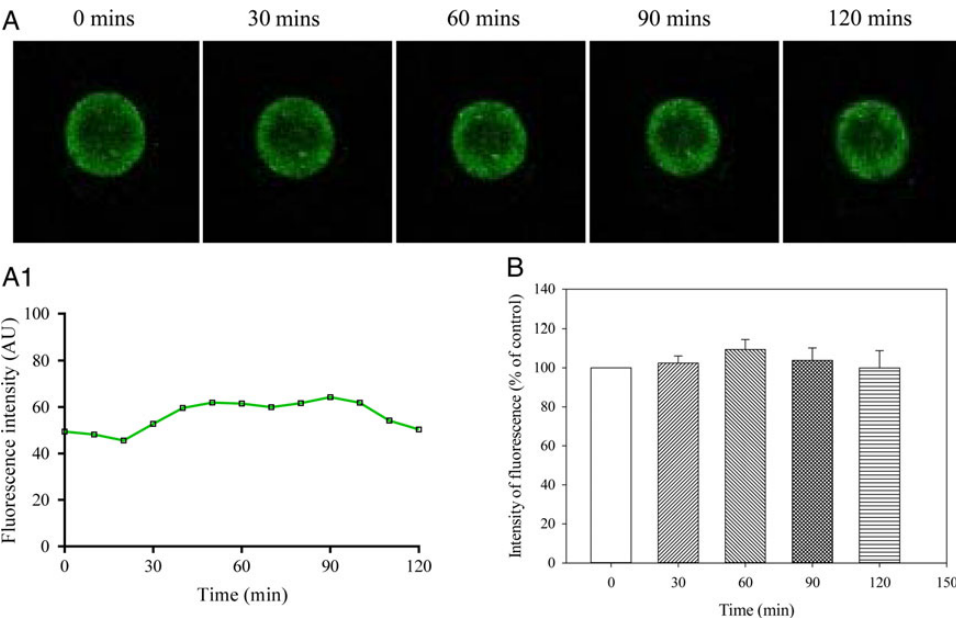


**Figure 2**  $\text{Ca}^{2+}$  increases over time in MII human oocytes. (A) Laser confocal images from an untreated oocyte (magnification  $\times 10$ ) at depicted time points. (A1) Changes in intensity of Fluo-3 fluorescence plotted as a function of time from oocytes in Fig. 2A. (B) Normalized intensity of Fluo-3 fluorescence in condition/time points corresponding to (A). Bars are mean  $\pm$  SEM ( $n = 19$  oocytes). \* $P < 0.05$ . Normalized intensity of Fluo-3 fluorescence in oocytes loaded with Fluo-3 at  $37^\circ\text{C}$  (C) and oocytes that were used for IVF or ICSI (D). Bars are mean  $\pm$  SEM ( $n = 7-23$  oocytes). \* $P < 0.05$  (ANOVA and t-test).





**Figure 3** DMSO does not modify  $\text{Ca}^{2+}$  increases over time in MII human oocytes. **(A)** Laser confocal images from an oocyte (imaged at cation  $\times 10$ ) at depicted time points in the presence of DMSO (vehicle used to dissolve all compounds) (0.1%). **(A1)** Changes in intensity of Fluo-3 fluorescence plotted as a function of time from oocytes in Fig. 3A. **(B)** Normalized intensity of Fluo-3 fluorescence in condition/time points corresponding to (A). Bars are mean  $\pm$  SEM ( $n = 8$  oocytes). \* $P < 0.05$  (ANOVA).



**Figure 4**  $\text{Ca}^{2+}$  does not increase over time in MII human oocytes in the presence of pinacidil. **(A)** Laser confocal images from an oocyte (magnification  $\times 10$ ) in the presence of pinacidil (100  $\mu\text{M}$ ) at depicted time points. **(A1)** Changes in intensity of Fluo-3 fluorescence plotted as a function of time from oocytes in Fig. 4A. **(B)** Normalized intensity of Fluo-3 fluorescence in condition/time points corresponding to (A). Bars are mean  $\pm$  SEM ( $n = 12$  oocytes).

## Glibenclamide inhibits the increase in $\text{Ca}^{2+}$ over time *in vitro* in human oocytes

In the experimental group treated with glibenclamide (100  $\mu\text{M}$ ), no statistically significant changes in intracellular  $\text{Ca}^{2+}$  were observed ( $P = 0.762$ ;  $n = 13$ ; Fig. 5). After 120 min, the intensity of fluorescence was increased by only  $5.9 \pm 10.9\%$  ( $n = 13$ ;  $P = 0.600$  when compared with fluorescence at the time point 0; Fig. 5).

## $\text{Ca}^{2+}$ increases in human oocytes exposed to DNP

DNP, an inhibitor of oxidative phosphorylation (Brady et al., 1996), was used to assess intracellular  $\text{Ca}^{2+}$  dynamics in human oocytes when exposed to severe metabolic stress. Intracellular  $\text{Ca}^{2+}$  was significantly increased in the presence of 100 mM DNP (Fig. 6). Intensity of fluorescence increased by  $25.9 \pm 6.7$  and  $105.7 \pm 29.5\%$  after 30 and 120 min, respectively ( $n = 10$ ;  $P = 0.004$  for both time points when compared with fluorescence at time point 0; Fig. 6).

## DNP induces increase in $\text{Ca}^{2+}$ in human oocytes in the presence of pinacidil

DNP increased intracellular levels of  $\text{Ca}^{2+}$  despite the presence of 100  $\mu\text{M}$  pinacidil ( $n = 5$ ;  $P = 0.012$ ; Fig. 7).

## Glibenclamide abolishes the increase in $\text{Ca}^{2+}$ in human oocytes exposed to DNP

In the presence of glibenclamide (10  $\mu\text{M}$ ), DNP (100 mM) did not increase the intracellular level of  $\text{Ca}^{2+}$  (Fig. 8). On the contrary, the

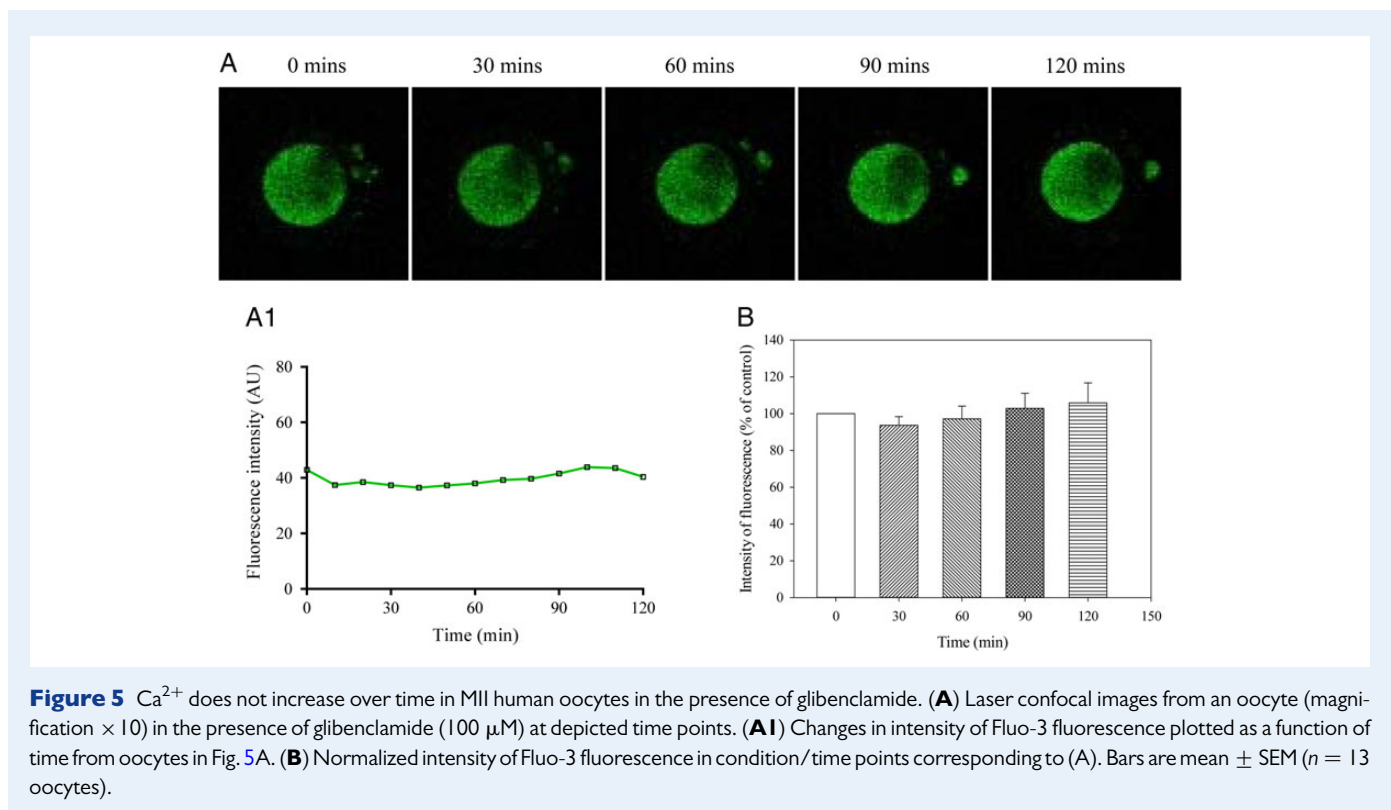
intensity levels of fluorescence were significantly lower over the time course of 2 h, as it decreased by  $52.3 \pm 10.3\%$  ( $P < 0.001$ ;  $n = 9$  for each; Fig. 8).

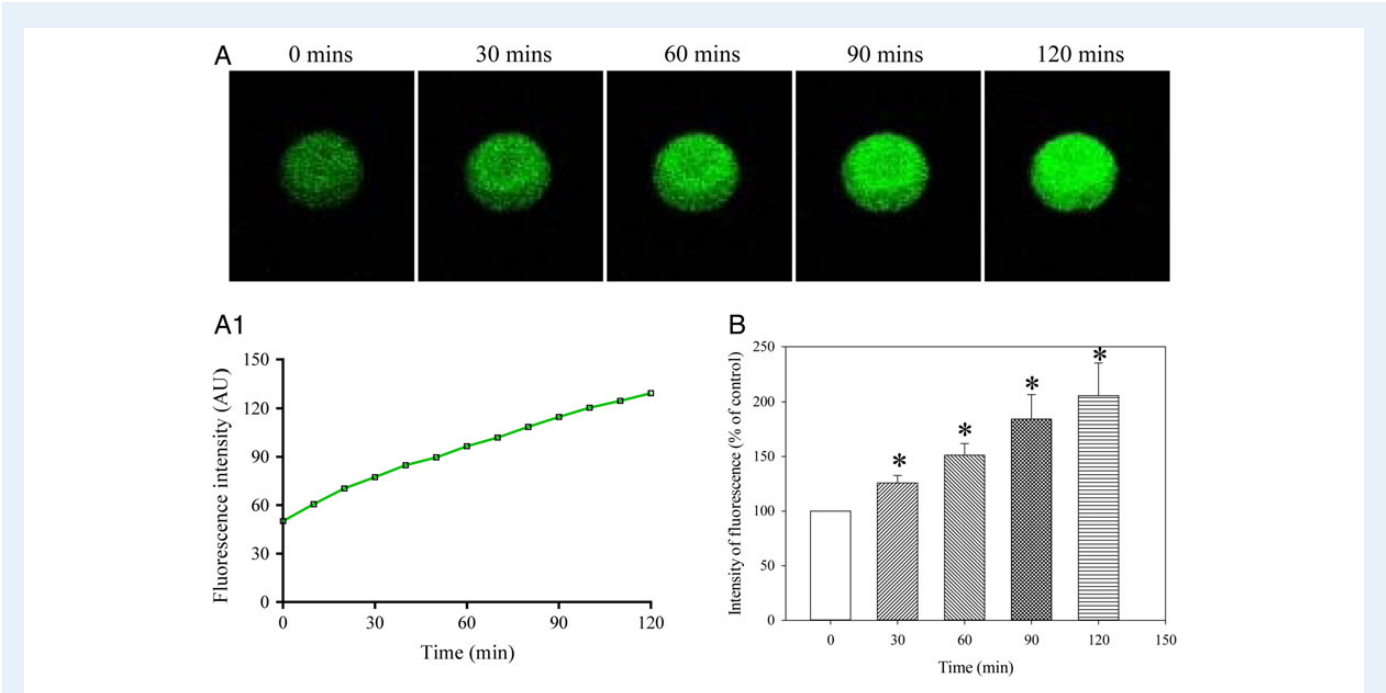
## Discussion

This study demonstrates that an increase in intracellular  $\text{Ca}^{2+}$  does occur in human oocytes when exposed to *in vitro* conditions and that this can be reduced and/or prevented by drugs targeting  $\text{K}_{\text{ATP}}$  channels.

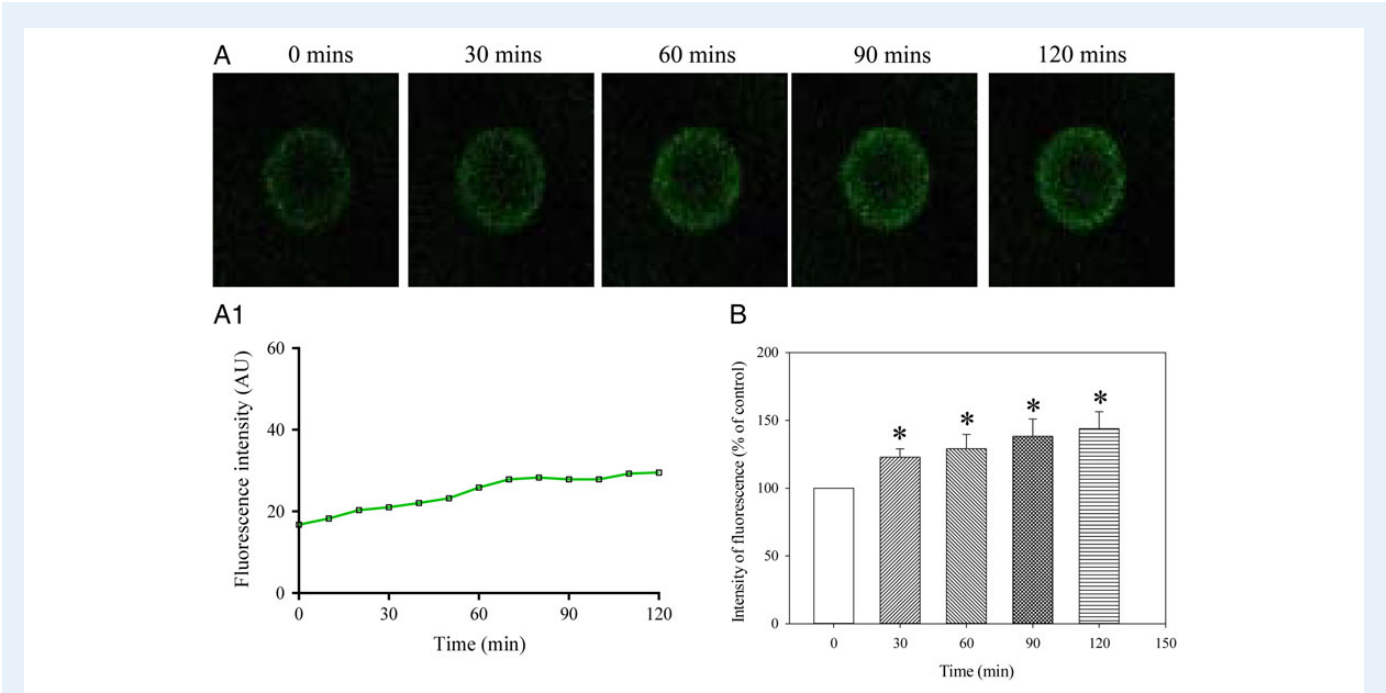
IVF and ICSI are standard interventions aimed to treat infertility. In order to carry out these techniques, it is necessary to remove an oocyte from its natural environment (the follicle) and prepare it for further procedures *in vitro*. Although in handling and manipulation of the oocytes in the laboratory, we aim to minimize external stresses, data from work on oocytes of experimental animals and some other cell types indicate that cellular stress could occur (Martín-Romero et al., 2008; Lord and Aitken, 2013). Due to clinical and ethical considerations, we were not able to monitor intracellular  $\text{Ca}^{2+}$  immediately after MII oocytes were collected as they were intended for therapeutic use and we obtained them only after IVF or ICSI were deemed unsuccessful. On average, cells were obtained for experimentation  $80.4 \pm 2.1$  h ( $n = 102$  oocytes) after collection, so we do not have information about  $\text{Ca}^{2+}$  dynamics prior to this period.

In this study, we have demonstrated a time-dependent increase in intracellular  $\text{Ca}^{2+}$  during monitoring of MII human oocytes for 2 h in *in vitro* conditions. This is the first report to suggest that  $\text{Ca}^{2+}$  homeostasis is challenged when oocytes are incubated under routine *in vitro* conditions, although this might be associated solely with protracted incubation. During incubation of oocytes, we have maintained conditions similar to physiological ones. The only exception was loading of cells with





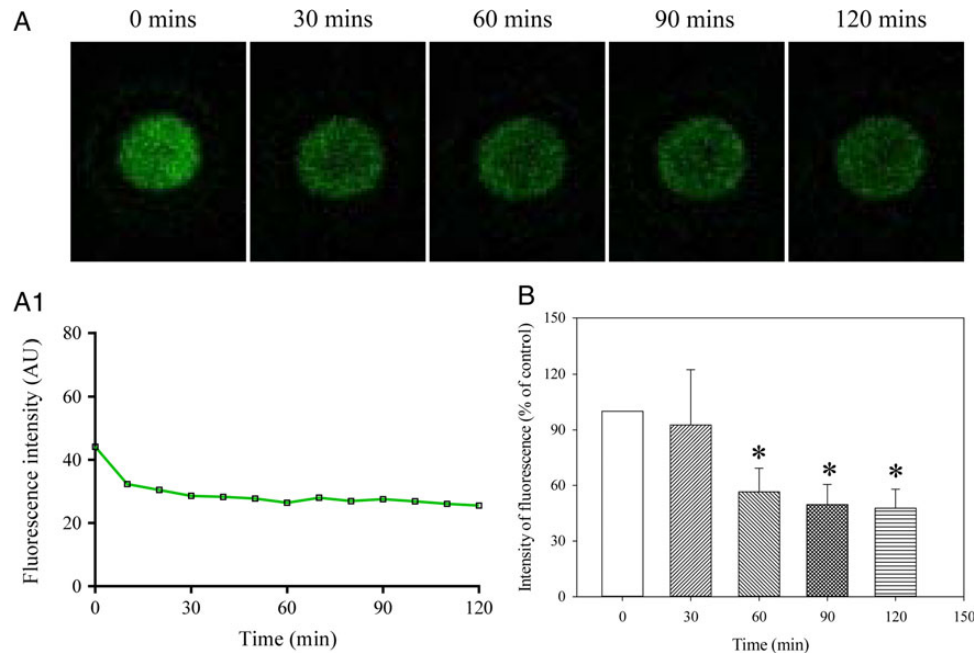
**Figure 6** Ca<sup>2+</sup> increases in MII human oocytes exposed to DNP. **(A)** Laser confocal images from an oocyte (magnification  $\times 10$ ) exposed to DNP (100  $\mu$ M) at depicted time points. **(A1)** Changes in intensity of Fluo-3 fluorescence plotted as a function of time from oocytes in Fig. 6A. **(B)** Average intensity of Fluo-3 fluorescence in condition/time points corresponding to (A). Bars are mean  $\pm$  SEM ( $n = 10$  oocytes). \* $P < 0.05$  (ANOVA).



**Figure 7** DNP induces an increase in Ca<sup>2+</sup> in MII human oocytes despite presence of pinacidil. **(A)** Laser confocal images from an oocyte (magnification  $\times 10$ ) exposed to DNP (100  $\mu$ M) plus pinacidil (100  $\mu$ M) at depicted time points. **(A1)** Changes in intensity of Fluo-3 fluorescence plotted as a function of time from oocytes in Fig. 7A. **(B)** Normalized intensity of Fluo-3 fluorescence in condition/time points corresponding to (A). Bars are mean  $\pm$  SEM ( $n = 5$  oocytes). \* $P < 0.05$ .

Ca<sup>2+</sup>-sensitive dye at room temperature. It has been previously reported that drops in temperature cause depolymerization of microtubules with increased risk of segregation errors and aneuploidy. Depolymerization of microtubules is influenced by Ca<sup>2+</sup> (Aman and Parks, 1994). However, loading of cells with Ca<sup>2+</sup> at 37°C did not increase the intracellular Ca<sup>2+</sup> under *in vitro* conditions, suggesting that





**Figure 8** Glibenclamide abolishes the DNP-induced increase in  $\text{Ca}^{2+}$  in MII human oocytes. **(A)** Laser confocal images from an oocyte (magnification  $\times 10$ ) exposed to DNP (100  $\mu\text{M}$ ) plus glibenclamide (100  $\mu\text{M}$ ) at depicted time points. **(A1)** Changes in intensity of Fluo-3 fluorescence plotted as a function of time from oocytes in Fig. 8A. **(B)** Normalized intensity of Fluo-3 fluorescence in condition/time points corresponding to (A). Bars are mean  $\pm$  SEM ( $n = 9$  oocytes). \* $P < 0.05$  (ANOVA).

temperature of the  $\text{Ca}^{2+}$ -sensitive dye did not affect  $\text{Ca}^{2+}$  dynamics. Subfertile women with endometriosis have elevated levels of systemic and intrafollicular oxidative stress markers (Andrade et al., 2010; Carvalho et al., 2012; Singh et al., 2013), thus oocytes arising from such patients could have potentially elevated intracellular  $\text{Ca}^{2+}$ . However, in the present study the overwhelming majority of donors were healthy, excluding the possibility that the oocytes we studied had altered  $\text{Ca}^{2+}$  homeostasis due to pathological process. It has been reported that ICSI is less successful in bovine and equine species due to altered  $\text{Ca}^{2+}$  dynamics in response to injection (Bedford et al., 2003; Malcuit et al., 2006). In addition to that, a difference in intracellular  $\text{Ca}^{2+}$  response was observed (Markoulaki et al., 2007) between oocytes subjected to ICSI or IVF procedures. Therefore, it was possible that oocytes subjected to ICSI or IVF respond differently to *in vitro* conditions. However, that was not the case as we did not find a statistically significant difference in  $\text{Ca}^{2+}$  dynamics between the two groups. Taken all together, our findings suggest that an increase in  $\text{Ca}^{2+}$  in oocytes persistently occurs under *in vitro* conditions.

$\text{Ca}^{2+}$  is a crucial signal for oocyte fertilization, as well as for early embryo development, and any adverse changes in  $\text{Ca}^{2+}$  homeostasis could feasibly decrease the probability of successful conception by a negative impact on oocyte quality (reviewed by Whitaker, 2006). This could represent an underlying reason for failure of IVF or ICSI. It seems that the duration of time that oocytes spending under *in vitro* conditions is an important factor determining success fertilization. Also, it has been reported that mice generated by IVF suffer from vascular dysfunction later in life and have a shortened lifespan (Rexhaj et al., 2013). As  $\text{Ca}^{2+}$  is a signalling molecule regulating a range of cellular functions (Clapham, 2007), it is possible that even when conception occurs it

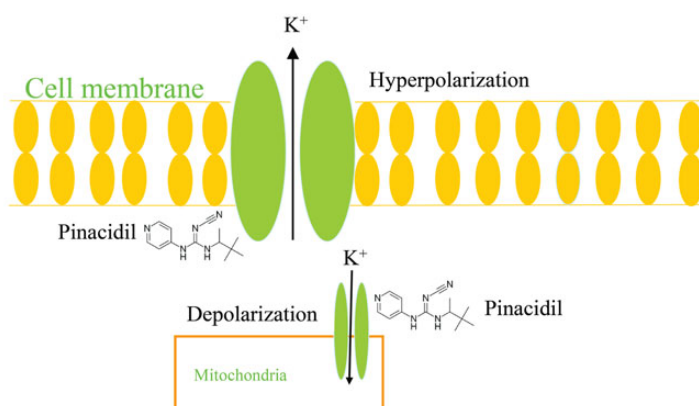
may have consequences for later life. There is no visible manifestation of increased  $\text{Ca}^{2+}$  in oocytes and the issue is that a  $\text{Ca}^{2+}$ -overloaded oocyte may be selected/used for IVF/ICSI resulting either in failure of fertilization or successful fertilization associated with health issues occurring later.

It has been recently shown that oocytes express  $\text{K}_{\text{ATP}}$  channels (Du et al., 2010).  $\text{K}_{\text{ATP}}$  channels link intracellular metabolic conditions with the membrane excitability (Nichols, 2006). We have hypothesized that activation of  $\text{K}_{\text{ATP}}$  channels could prevent  $\text{Ca}^{2+}$  loading in oocytes occurring *in vitro*. Therefore, we have tested the effect of pinacidil, a well-established  $\text{K}_{\text{ATP}}$  channel-opening drug (Nichols, 2006). In the presence of pinacidil,  $\text{Ca}^{2+}$  levels remained steady suggesting that the activation of  $\text{K}_{\text{ATP}}$  channels prevents  $\text{Ca}^{2+}$  loading induced by *in vitro* conditions. It has been shown that the opening of  $\text{K}_{\text{ATP}}$  channels clamps the membrane potential at a more negative value, keeping voltage-dependent  $\text{Ca}^{2+}$  entry pathways closed and preventing influx of  $\text{Ca}^{2+}$  (Jovanović and Jovanović, 2001). Taking into consideration that influx of  $\text{Ca}^{2+}$  is a crucial part of  $\text{Ca}^{2+}$  signalling in mammalian oocytes (reviewed by Gómez-Fernández et al., 2012), the observed inhibition of  $\text{Ca}^{2+}$  loading in human oocytes is in agreement with such an effect of  $\text{K}_{\text{ATP}}$  channel opening. On the other hand, glibenclamide is an oral antidiabetic known to inhibit  $\text{K}_{\text{ATP}}$  channels opening (reviewed by Abdelmoneim et al., 2012). In cells where  $\text{K}_{\text{ATP}}$  channels are physiologically closed application of this drug usually has no significant effects (Brady et al., 1996; Budas et al., 2004), while in cells where  $\text{K}_{\text{ATP}}$  channels are open, glibenclamide often induces membrane depolarization and  $\text{Ca}^{2+}$  loading, even leading to cell death (Brady et al., 1996; Budas et al., 2004; Abdelmoneim et al., 2012). Surprisingly, we have found that glibenclamide actually prevents  $\text{Ca}^{2+}$  loading induced by *in vitro* conditions in human MII oocytes.

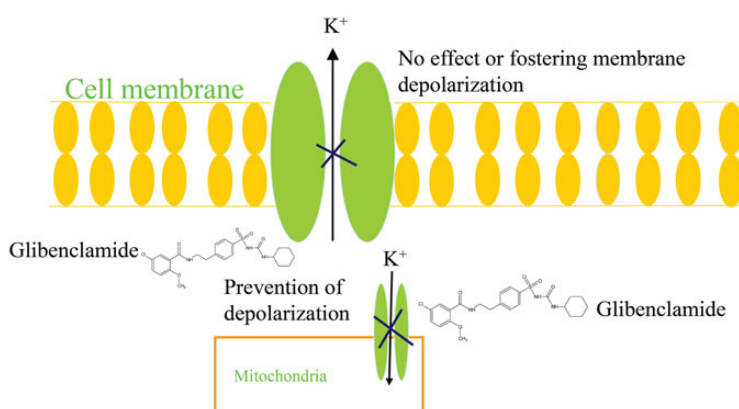
Structurally,  $\text{K}_{\text{ATP}}$  channels are composed of a pore-forming inward rectifier, Kir6.1 or Kir6.2, and a regulatory, ATP-binding subunit, SUR1, SUR2A or SUR2B. It is generally accepted that four Kir6.x and four SURx are physically associated with each other to form functional  $\text{K}_{\text{ATP}}$  channels (reviewed by Nichols, 2006). In our previous study, we have suggested that the  $\text{K}_{\text{ATP}}$  channel composition in human MII oocytes is the SUR2A/Kir6.1 combination (Du *et al.*, 2010). Kir6.1 is a pore-forming channel subunit in the vascular form of  $\text{K}_{\text{ATP}}$  channels (Nichols, 2006). Glibenclamide normally increases intracellular  $\text{Ca}^{2+}$  (Brady *et al.*, 1996; Budas *et al.*, 2004; Abdelmoneim *et al.*, 2012). The only finding similar to ours was reported on vascular smooth muscle cells where glibenclamide inhibited the increase in  $\text{Ca}^{2+}$  evoked by high extracellular  $\text{K}^+$  and noradrenaline (Yoshitake *et al.*, 1991)

despite inducing membrane depolarization (Wilson *et al.*, 2000). It is certainly intriguing that compounds inhibiting and activating  $\text{K}_{\text{ATP}}$  channels can act in the same manner to prevent  $\text{Ca}^{2+}$  loading induced by *in vitro* conditions. One possible explanation is that prevention of the  $\text{Ca}^{2+}$  increase is due to the differential effects of pinacidil and glibenclamide on different populations of  $\text{K}_{\text{ATP}}$  channels in oocytes. As an example, the activation of plasmalemmal  $\text{K}_{\text{ATP}}$  channels prevents  $\text{Ca}^{2+}$  influx by clamping the membrane potential (Jovanović and Jovanović, 2001), but the inhibition of  $\text{K}_{\text{ATP}}$  channels localized in mitochondria inhibits release of their  $\text{Ca}^{2+}$  (Holmuhamedov *et al.*, 1998). Thus, it is quite plausible that a decrease in  $\text{Ca}^{2+}$  induced by pinacidil is due to inhibition of membrane depolarization, while a decrease in  $\text{Ca}^{2+}$  induced by glibenclamide could be due to inhibition of mitochondrial release of  $\text{Ca}^{2+}$  (Fig. 9).

**A** Protection of oocytes by pinacidil can be explained primarily by inhibition of stress-induced membrane depolarisation and influx of  $\text{Ca}^{2+}$ . Functional consequences of activation of subcellular/mitochondrial  $\text{K}_{\text{ATP}}$  channels and resulting mitochondrial membrane depolarisation when oocytes are yet unknown.



**B** Protection of oocytes by glibenclamide can be explained by inhibition of subcellular (mitochondrial as an example)  $\text{K}_{\text{ATP}}$  channels and consequent prevention of  $\text{Ca}^{2+}$  release from internal stores (such as mitochondria). Inhibition of plasmalemmal  $\text{K}_{\text{ATP}}$  channels could foster membrane depolarisation or be without effect.



**Figure 9** A possible mechanism underlying cytoprotective action of pinacidil and glibenclamide in human oocytes. Cartoon summarizing possible underlying mechanisms mediating pinacidil- (**A**) and glibenclamide-induced (**B**) cytoprotection in MII human oocytes based on the findings from the present study as well as findings from previous studies that have investigated  $\text{K}_{\text{ATP}}$  channels in oocytes and other cell types (Brady *et al.*, 1996; Holmuhamedov *et al.*, 1998; Jovanović *et al.*, 1999; Jovanović and Jovanović, 2001; Du *et al.*, 2010).

DNP is an inhibitor of oxidative phosphorylation. It induces severe metabolic stress (Brady et al., 1996; Du et al., 2010), but also activates  $K_{ATP}$  channels in human oocytes (Du et al., 2010). When exposed to DNP, intracellular levels of  $Ca^{2+}$  increased in human oocytes, but this increase was not dramatically different to those observed when oocytes were left under *in vitro* conditions alone. It is possible that either (i) DNP has no a major effect on human oocytes or (ii) that human oocytes had passed a threshold of *in vitro* stress. In pig oocytes, exposure to *in vitro* conditions alone does not affect intracellular levels of  $Ca^{2+}$ , while exposure to DNP significantly increases intracellular  $Ca^{2+}$  (unpublished data) suggesting that DNP induces significant stress in mammalian oocytes (Grundlingh et al., 2011). We conclude that the inability of DNP to further increase  $Ca^{2+}$  in human oocytes is probably due to the already high sensitivity and stress of these cells following extended *in vitro* conditions. Pinacidil inhibited  $Ca^{2+}$  loading induced solely by *in vitro* conditions, but was not as efficient on DNP-induced  $Ca^{2+}$  loading. In contrast, glibenclamide maintained  $Ca^{2+}$  homeostasis in oocytes despite cells being challenged by DNP. It seems that glibenclamide, an antagonist of  $K_{ATP}$  channels, is more efficient as cytoprotective agent in human oocytes than pinacidil, a  $K_{ATP}$  channel opener.

In principle, compounds that protect oocytes against stress occurring in *in vitro* conditions have a potential to improve the rate of fertilization *in vitro* and embryo quality. This study suggests that compounds targeting  $K_{ATP}$  channels should be tested as a means for improving the outcome of ART.

## Conclusion

In conclusion, this study has demonstrated that human oocytes maintain  $Ca^{2+}$  homeostasis with difficulty when exposed to routine *in vitro* conditions, which could interfere with fertilization. Both inhibition and activation of  $K_{ATP}$  channels is useful for maintaining  $Ca^{2+}$  homeostasis in oocytes under *in vitro* conditions. It seems that inhibition of  $K_{ATP}$  channels is a particularly efficient strategy in protecting human oocytes against stress. Such a strategy should be tested in an ART setting to examine if it leads to an improve rate of success.

## Authors' roles

All authors have contributed to the study design, revised and approved the manuscript. In addition to that, G.F., N.D., N.K., A.M., M.G., E.D., E.B., O.A.M., S.G.B., A.M.M., S.J.M.S. and K.M.W. were involved in oocyte and patients data collection, whilst G.F., N.D., N.K., A.M. and A.M.M. have performed experiments using these oocytes. A.J. has designed and supervised the study, drafted and approved the manuscript.

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## Conflict of interest

None declared.

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